Comparison of low and high DNAPurity for quantitative detection of ratio Mitochondrial and Nucleus DNA among Drug-treated HIV Patients by Real-time PCR

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Comparison of low and high DNA Purity for quantitative detection of ratio Mitochondrial and Nucleus DNA among Drug-treated HIV Patients by Real-time PCR

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Abstract. Antiretroviral in nucleoside reverse transcriptase inhibitors (NRTIs) can inhibit the mitochondrial enzyme polymerase γ, in-vitro and in-vivo to halt the extension of DNA known as the terminal chain terminator. The technique used is measuring the amount of mitochondrial and nucleus DNA in cells by real-time polymerase chain reaction (qPCR). Although the result is not consistent because of some factors. We optimized the mitochondrial and nucleus DNA quantification methods using high and low purity DNA by qPCR. HIV-1-infected individuals were recruited from Gianyar and Denpasar, Bali. HIV-1 DNA was extracted from peripheral blood mononuclear cells (PBMCs). Quality and quantity of DNA were measured by spectrofotometer. Quantification of mitochondrial and nucleus DNA using standard curve by qPCR. Optimizing primer and annealing temperature for quantification of mitochondrial DNA revealed that single peak in Tm 64,5°C for sets of primer CCOI and primer RNR1. In this study, standard curve was determined copy number of mitochondrial DNA. From standard curve, the high DNA purity was increased the PCR amplification efficiency. Otherwise, the low DNA purity was decreased the PCR amplification efficiency. Concentration and purity of DNA are influenced by PCR amplification efficiency. Therefore, we should be considered sample quality and technique of pipetting.

1. Introduction

Antiretroviral is recommended for everyone who has *human immunodeficiency virus* (HIV) infection and it has greatly improved the patient's survival. However, the importance of aging-related co-morbidities has increased such as cardiovascular event [1], metabolic abnormalities [2] or bone density disturbance [3,4]. Nucleosides reverse transcriptase inhibitors (NRTI) are included in most antiretroviral treatment (ART) regimens. Exposure to NRTI is associated with a diffuse spectrum of adverse metabolic reactions consistent with mitochondrial toxicity. These side effects were initially thought to be primarily due to inhibition of the mitochondrial DNA gamma polymer (mtDNA). Subsequent work suggests that NRTI may cause mitochondrial dysfunction through a variety of mechanisms, which may vary depending on the specific NRTI and cell type. Because the clinical symptoms associated with mitochondrial toxicity are variable and range in severity, there would be a substantial clinical utility to a non-invasive diagnostic assay to evaluate mitochondrial toxicity in HIV-infected individuals on ART [5].

The best methods to detect mitochondrial and nucleus DNA by realtime PCR (qPCR). PCR efficiency is an important factor in the reliability of the results. PCR amplification efficiency is the rate at which a PCR amplicon is generated, and is commonly expressed as a percentage value. In theory, every cycle of PCR doubles the amplicon amount. This represents 100% efficiency. During the log phase, PCR efficiency should be as close to 100% as possible. Efficiencies of more than 100% may indicate sample quality or pipetting problems. Addition, the quantity and quality of the DNA extracted are mostly influenced by many factors such as sampling techniques, sample size, matrix type, inhibitors substances, the grade of damage, and the fragment length of the DNA [6,7]. This study aimed to determine the best quantitative detection of mitochondrial and nucleus DNA methods in high and low DNA purity from HIV patients by qPCR.

2. Material and methods

2.1. Sample collection

Fifty-eight HIV-infected individuals were recruited from the Sinta HIV Clinic of Sanjiwani Hospital in Gianyar regency and Puskesmas Denpasar Selatan II at Denpasar municipality in Bali, Indonesia. Eight milliliters whole blood samples were collected from study participants. This study was conducted with approvals from the Medical Research Ethics Committees of the Faculty of Medicine, Udayana University, and Airlangga University. All study participants were enrolled after providing written informed consents. Fifty-eight samples were amplified to determine Mitochondrial DNA and Nucleus DNA. For curve standart using two samples which have low and high concentration of DNA.

2.2. DNA extraction

Ten milliliters of ethylene diamine tetra acetic acid (EDTA) anti-coagulated peripheral blood was collected. Plasma was collected from peripheral blood by centrifugation for 10 minutes at 2,000 rpm. Also, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA). DNA was extracted from PBMC using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich).

2.3. DNA concentration and Purity

The DNA concentrations were determined by measuring the absorbance at 260 nm wavelength (A260) and 280 nm wavelenght (A280) using spectrophotometer (Nanodrop, Thermoscientific). Purity was determined by calculating the ratio of absorbance at 260 nm and the absorbance at 280 nm (A260/ A280).

2.4. Real-time PCR Assay

The presence of mt-DNA and n-DNA was performed by CFX96 Touch Real-time Detection System (BIO-RAD). The validity of real-time PCR assay is one that has been assessed for the optimal range of primer annealing temperatures, reaction efficiency, and specificity using a standard set of samples (1). The reaction was setup using EVA Green SMX 200R kit, Bio-Rad, Laboratories USA. The reaction contained five µl master mix (2x), two µl DNA template (2 ng/µl), one µl forward primer (10 µM), one µl reverse primer (10 µM) and PCR grade water to the final volume of 20 ml. To amplify the presence of n-DNA, the primers RNR1 sense 5'-ATCCTTCGATGTCGGC-3' and RNR1 antisense 5'-AGCACATACACCAAATGTCT-3' (2). Also, to amplify the presence of mt-DNA, the primers CCOI sense 5'-TTCGCCGACCGTTGACTATT-3' and CCOI antisense 5'-AAGATTATTACAAATGCATGGGC-3' (3). The Real-time PCR conditions were as follows. For mt-DNA and n-DNA amplification, one cycle of 3 min at 95°C for denaturation; 40 cycles of 10sec at 95°C for denaturation, 30 secs at 64,5°C for annealing and 1 min at 72°C for extension; and melt curve analysis consisted of 64,5°C to 95°Cfor 5 sec with increment 0.5°C.

3. Results and discussions

Nucleic acid quantification is an important role for detection of mitochondrial and nucleus DNA by qPCR [8,9]. DNA concentration and purity are determined by measuring the ratio of UV absorbance at 260 nm and 280 nm. In this study, we extracted DNA from two different PBMCs and the value of A_{260}/A_{280} showed the difference between sample 1 and sample 2 with the same DNA extraction methods. Sample 1 showed high purity, and sample 2 showed low purity. The differences in quality and quantity from DNA extracted is influenced by amplification process.

Table 1. Concentration and Purity of DNA extractions.

Sample No.	Concentration (ng/µl)	A_{260}/A_{280}
1	166	1.86
2	11.5	1.17

For amplification of DNA, we should be optimize the primer and annealing temperatures to determine the specificity using a standard set of samples by gradient temperature. To find the optimal annealing temperature is suitable for a range of below and above melting temperature (Tm) of the primers. In this study, the gradient temperature using primer CCOI and RNR1 about a range in temperature 52°C; 53°C; 55°C; 57,9°C; 61,5°C; 64,5°C; 66,2°C; 67°C. The non-specific and specific product using primer CCOI and RNR1 can be identified by melt-curve analysis, a specific product is single peak, and non-specific product is more than one peak. The specific product is the single peak with a temperature melting (Tm) 64,5°C for sets of primer CCOI and sets of primer RNR1 as shown in Figure 1.

Detection of mitochondrial and nucleus DNA among HIV-1 infected patients using sets of primer CCOI and sets of primer RNR1 using standard curve. Quantification using standard curve, the quantity (copy number or unit mass) of the unknown sample is interpolated from a range of standard a known quantity. In this study, we used range of standard concentration 300000; 30000;3000;300;30 (copy number) correlation with 495000;49500;4950;495;49,5 (pg/µl). We compared using different DNA purity, high and low DNA purity. Unknown samples are assayed with the standards in same experimental. The standard curve constructed from the diluted standard can be used to determine the target quantity in the unknown sample by interpolation. From standard curve, we got the equation for linear regressions line $y = mx + b$. The equations of linear regressions line in Figure 2 was used to calculate the copy number of unknown sample. The copy numbers of mitochondrial and nucleus DNA were determined using their C_T values (data is not shown).

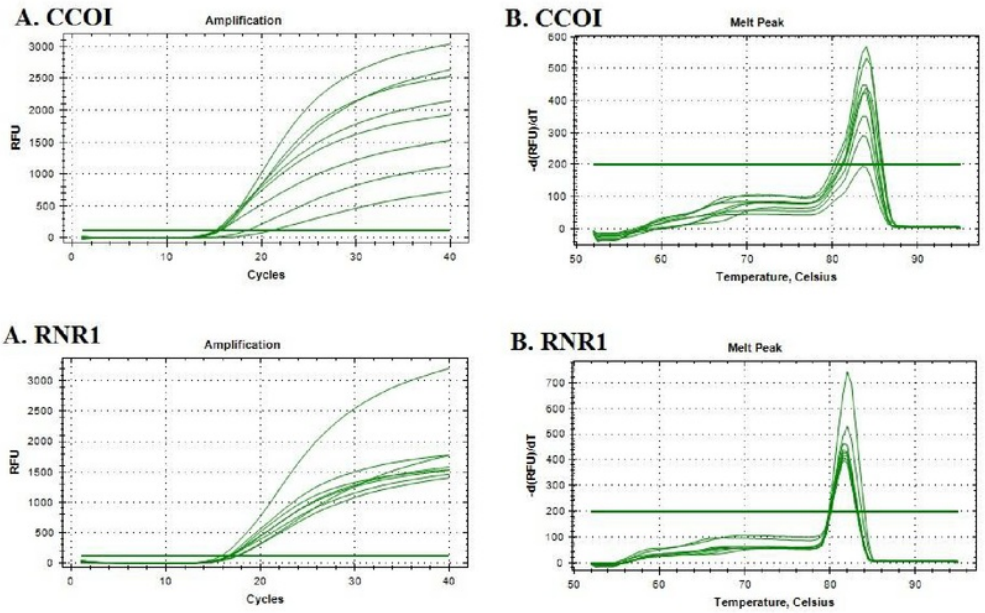
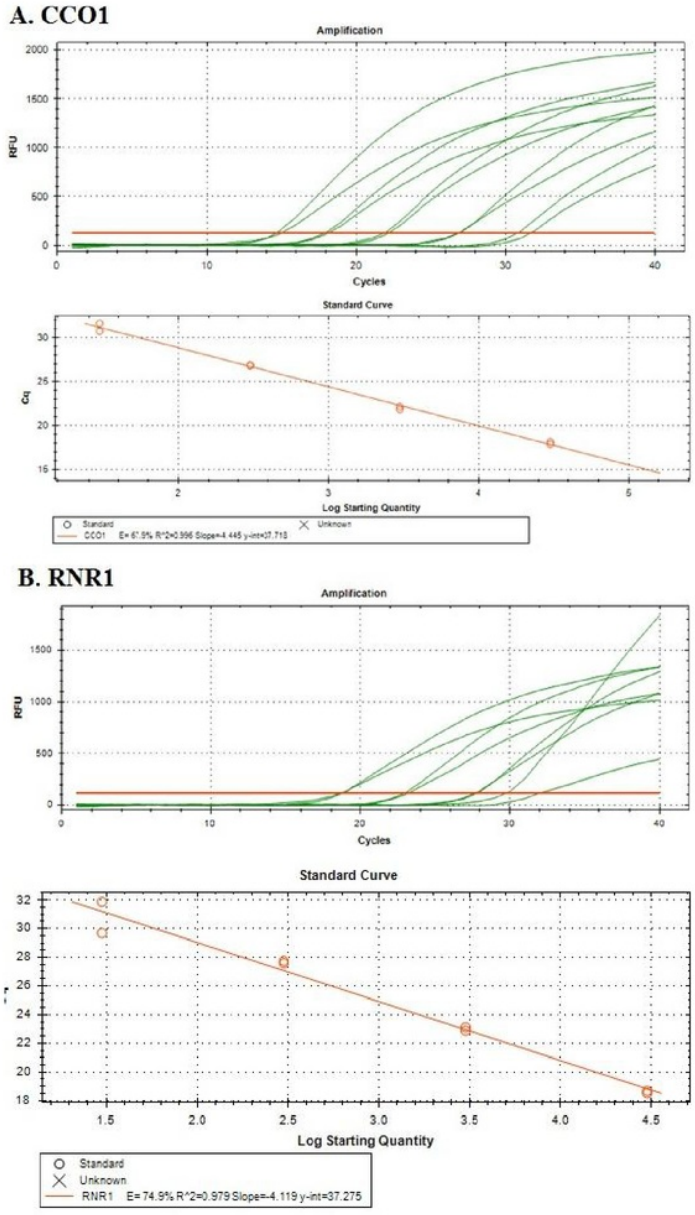
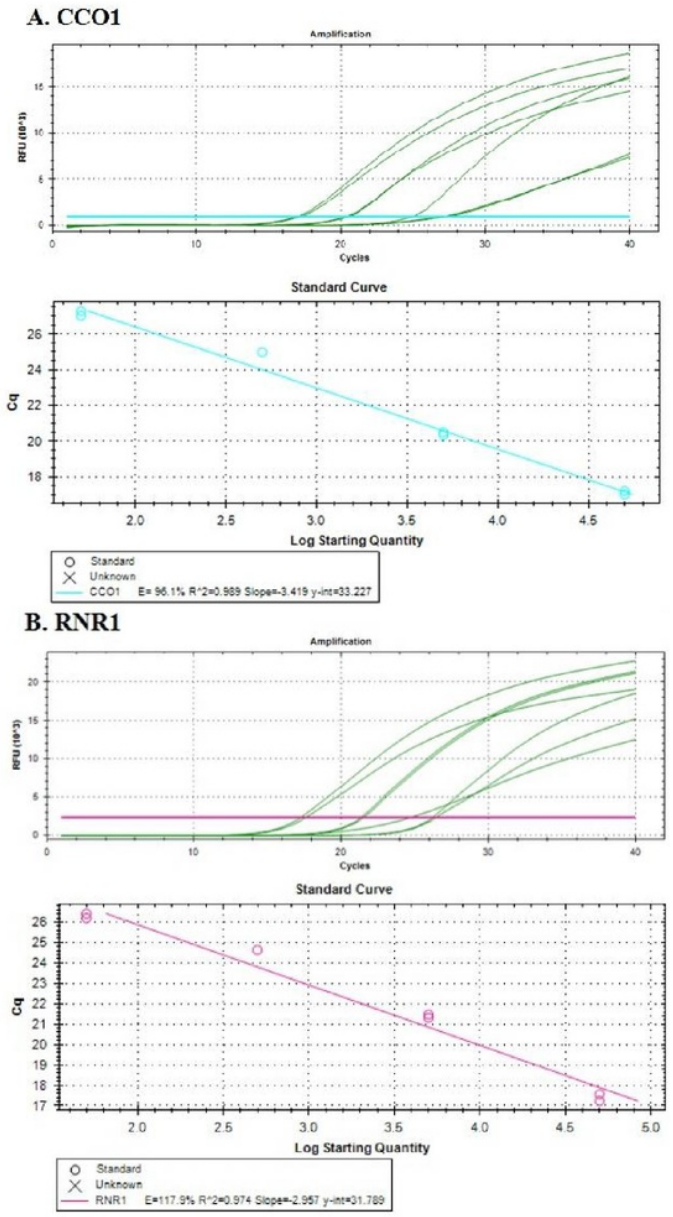


Figure 1. Validation of qPCR primers. (A) qPCR is performed at a range of annealing temperatures using a thermal gradient block. Amplification profiles indicate that the most efficient amplification occurs at the four lowest annealing temperatures between 52°C and 67 °C where the curves are at the lowest Cq; (B) a single peakon the melt curve analysis indicates a single PCR product.

PCR amplification efficiency close to 100% is the best parameter assay. In preliminery study, you should require for an amplification efficiency of 90 – 105%. Low reactions efficiency may be caused by poor primer design or by suboptimal reactions conditions. The presence of inhibitor can also result in an apparent increase in efficiency. The high concentration and purity DNA can increase the level of inhibitor, so the C_T values are also increased [10-12]. It similar with this study the purity DNA related to PCR amplification efficiency, the high DNA purity still increase gradually the amplification efficiency. For high DNA purity using sets of primer CCOI and RNR1, the amplification efficiency is 96,1% and 117,9%. But for low DNA purity using sets of primer CCOI and RNR1, the amplification efficiency is 67,9% and 74,9%1 (Figure 2).


A. CCO1
Amplification
RFU
Cycles
Standard Curve
Cq
Log Starting Quantity
Standard
Unknown
CCO1 E= 67.5% R^2=0.996 Slope=-4.445 y-int=37.718
B. RNR1
Amplification
RFU
Cycles
Standard Curve
Log Starting Quantity
Standard
Unknown
RNR1 E= 74.9% R^2=0.979 Slope=-4.119 y-int=37.275

(1)



(2)

Figure 2. Typical real-time polymerase-chain-reaction standard curves generated for quantitative detection of ratio Mitochondrial and Nucleus DNA among Drug-treated HIV Patients. The numbers (30 to 30,000) shown in the standard curve for quantitative detection of ratio Mitochondrial and Nucleus DNA (1) Low DNA purity and (2) High DNA purity

4. Conclusions

Real-time PCR is best methods for quantification of mitochondrial and nuclear DNA using a standard curve. DNA purity affects the qPCR process; low purity leads to a reduction of product amplification so that the amplification efficiency value is low.

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